

## The characteristics of immobilized granular sludge in the laboratory-scale stable partial nitrification-Anammox aquaculture water reactors

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### ABSTRACT

Immobilized granular sludge is of key importance for highly effective operation of partial nitrification and anaerobic ammonium oxidation (PN-Anammox) reactors. An observation and analysis on the composition of immobilized granular sludge in each reactor was conducted by using scanning electron microscopy (SEM) and fluorescence *in situ* hybridization. It was indicated that the diversity of microbial composition was significant among the PN and Anammox reactor. The result shows that the understanding of microbiological characteristics of immobilized granular sludge in PN and Anammox reactors is helpful for cultivating granular sludge, which ensures the effective operation of the reactor.

**Key words** | anaerobic ammonium oxidation, immobilized granular sludge, microbiological characteristics, partial nitrification-Anammox reactor

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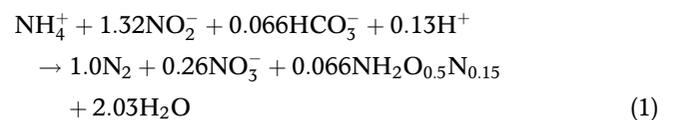
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### INTRODUCTION

Anaerobic ammonium oxidation (Anammox) is a biological process in which ammonium is converted to dinitrogen under anoxic conditions with nitrite as the electron acceptor (Equation (1)) (Mulder *et al.* 1995). The Anammox process has been recognized as a promising cost-effective and low energy alternative to the conventional nitrification-denitrification processes, due to a significant reduction of aeration and alkalinity for nitrification and organic carbon for denitrification (Van Dongen *et al.* 2001; Kartal *et al.* 2010).

Generally, ammonium is a main nitrogen compound in most wastewater. In nitrogen removal via the Anammox process, ammonium in wastewater must be partly pre-oxidized to nitrite (i.e. partial nitrification (PN)), but not to nitrate, before feeding into the Anammox process. The produced nitrite together with the remaining ammonium is then

converted to denitrogen gas (N<sub>2</sub>) in the Anammox process.



This sequential PN and Anammox process can be performed in two different reactors or in a single reactor. Although the PN-Anammox process is a completely autotrophic process, it has been successfully applied to treatments of sewage sludge digester liquid (Van Dongen *et al.* 2001; Fux *et al.* 2002) and livestock manure digester liquid (Yamamoto *et al.* 2008, 2011). The drawback of this process is the low growth rate of the bacteria involved (Isaka *et al.* 2006). Therefore, the Anammox reactors are often operated at long solids retention times in order to accumulate the necessary biomass in the system

(Fernández *et al.* 2008; López *et al.* 2008). The granulation of the Anammox biomass offers an effective strategy to retain the biomass in Anammox reactors due to its good settleability (Arrojo *et al.* 2006, 2008; Van der Star *et al.* 2007; Lu *et al.* 2012).

In this study, a laboratory-scale PN-Anammox process was developed in two separate reactors. The reactor was inoculated with immobilized granular sludge. The structure and micro-ecology characteristics of the anaerobic granular sludge in each separate reactor were observed and analyzed using the scanning electron microscopy (SEM) and fluorescence *in situ* hybridization (FISH) techniques. The objective of this work was to investigate the characteristics of Anammox granules so as to further improve the performance of high-rate PN and Anammox reactors.

## MATERIAL AND METHODS

### Inoculated sludge

The sequential PN and Anammox process was performed in two different reactors. The sludge from an aquafarm was used to cultivate inoculated sludge. The ZSM-5 zeolite was used as a carrier in this project. The cultivated nitrification and Anammox bacteria were fixed to the carrier in the reactor, respectively. The reactor was inoculated with immobilized granular sludge. The sludge concentration in the reactor after inoculation was approximately 3.3 g/L.

### Characteristics of raw wastewater

The feed used in this study was aquaculture wastewater, collected from an aquafarm at Yangzhou in Jiangsu, China. The composition of the raw wastewater was: pH 7.2–8.1, chemical oxygen demand (COD) 150–200 mg/L,  $\text{NH}_4^+\text{-N}$  80–100 mg/L,  $\text{NO}_3^-\text{-N}$  and  $\text{NO}_2^-\text{-N}$  < 2 mg/L, suspended solids (SS) 200–256 mg/L, total nitrogen (TN) 83–105 mg/L.

### Configuration of reactor

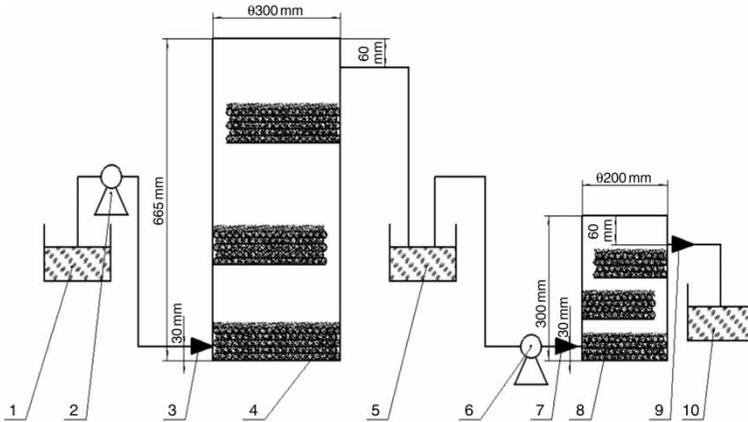
An aeration tank was used as the PN reactor. The sludge from an aquafarm was pre-cultured with a synthetic nutrient medium (components:  $(\text{NH}_4)_2\text{SO}_4$  2 g, NaCl 0.3 g,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.03 g,  $\text{K}_2\text{HPO}_4$  1 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.03 g,

$\text{NaHCO}_3$  1.6 g,  $\text{H}_2\text{O}$  1 L, pH 7.2, T 37 °C) for 20 days. The nitrification bacteria were fixed to the ZSM-5 zeolite then inoculated into the PN reactor. The reactor had an inner diameter of 30 cm and a height of 66.5 cm. It was constructed from perspex, with a valid volume of 28.5 L. The temperature was maintained at  $26 \pm 1$  °C. The dissolved oxygen (DO) was approximately 0.5–0.8 mg/L. The pH was controlled between 7.0 and 8.5.

An anaerobic tank was used as the Anammox reactor (Figure 1), which was inoculated with immobilized granular Anammox biomass taken from a full-scale upflow anaerobic sludge blanket reactor operating for treatment of pulp and paper wastewater in Jiangsu Province, China. The Anammox reactor had an inner diameter of 20 cm and a height of 36.5 cm. It was constructed from perspex, with a valid volume of 9.5 L. The temperature was maintained at  $32 \pm 1$  °C. The reactor was divided into three equal compartments by horizontal clapboard.

### Analytical methods

To monitor the performance of both reactors,  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_2^-\text{-N}$ , and  $\text{NO}_3^-\text{-N}$  in the influent and effluent were regularly measured by using ion-exchange chromatography (DX-100, DIONEX, CA, USA) with an IonPac CS3 cation column and IonPac AS9 anion column after filtration with 0.2- $\mu\text{m}$  pore size membranes (Advantec, Tokyo, Japan). DO concentration in the PN reactor was measured by using a DO meter (DO-5Z, KRK, Japan). Volatile suspended solids were measured according to the standard methods. The concentration of free ammonia (FA;  $\text{NH}_3$ ) was calculated as a function of pH, temperature and total ammonium nitrogen. The COD of the influent and effluent was determined according to *Standard Methods* (APHA/AWWA/WEF 2005). SEM showed the bacteria of granular sludge existed in the PN and Anammox reactor on day 90 after start-up, and on day 320. We utilized the AOB bacteria (Nso190, Nsv443 and Nsm156) and Anammox (Amx368) probes labeled with FITC (yellow) and Cy3 (red), respectively. Imaging using confocal laser scanning microscopy revealed a high density of cells growing in clusters and emitting red and yellow fluorescence, indicating the microbial composition of the granules (Figure 6(a)). The procedures of granule fixation, sectioning, and FISH were performed



**Figure 1** | Schematic diagram of the PN-Anammox reactor. (1) Feed tank; (2) peristaltic pump 1; (3) influent to PN reactor; (4) AOB granular sludge; (5) flow equalizing tank; (6) peristaltic pump 2; (7) influent to Anammox reactor; (8) Anammox granular sludge; (9) effluent; (10) effluent tank.

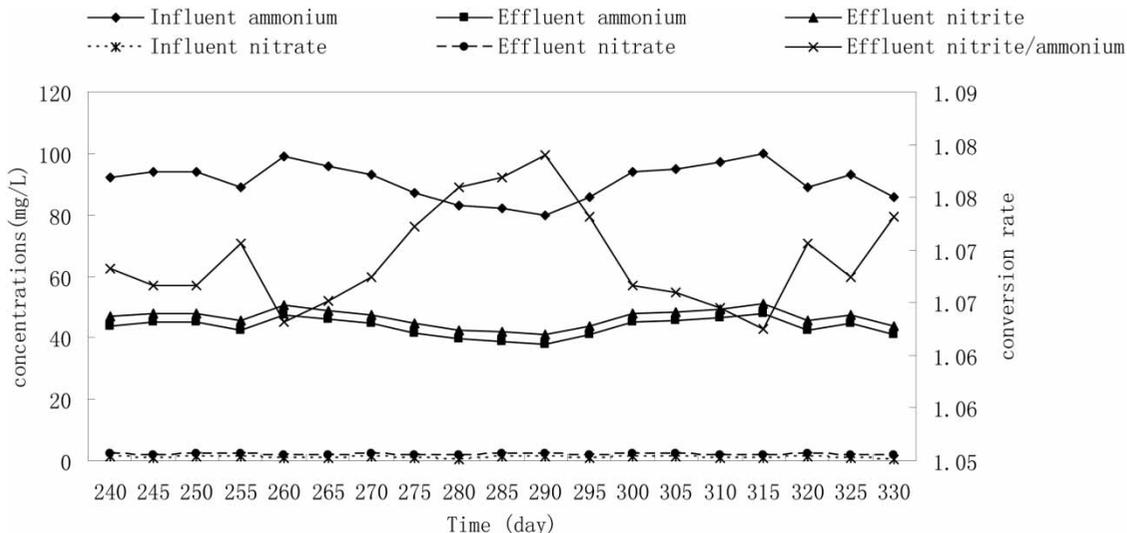
according to the protocol described previously (Sekiguchi *et al.* 1998). The fluorescence labels of the oligonucleotide probes used in this study are listed in the literature (Qiao *et al.* 2013; Wang *et al.* 2013).

## RESULTS AND DISCUSSION

### Performance of the PN-Anammox process

After establishing stable PN, the PN reactor was connected to an Anammox reactor. The effluent from the PN reactor

was then introduced into the Anammox reactor via a flow equalizing tank (volume 500 mL), where DO carried over from the PN reactor was removed, and the pH was adjusted. The flow rate to the PN reactor was set to obtain a hydraulic retention time (HRT) of 4 h in the Anammox reactor. The performance of the PN-Anammox reactor in a steady-state condition was carried out for 90 d (starting from the 240th day), accompanied with a consistent HRT of 12 h and an influent  $\text{HCO}_3^-/\text{NH}_4^+$  molar ratio of 1:1 in the PN reactor. The DO in the PN system was maintained within the range of 0.5–0.8 mg/L. The nitrification performance over time is shown in Figure 2. Half of the amount of ammonium in the



**Figure 2** | Profiles of influent and effluent nitrogen concentrations, and the ratio of nitrite/ammonium in the PN process.

influent was oxidized to nitrite in the PN reactor. The ammonium and nitrite concentrations in the PN reactor effluent were in the range of 40–50 mg-N/L, respectively, giving a ratio of ammonium to nitrite concentration of approximately 1:1.07. The ratio of ammonium to nitrite concentration of approximately 1:1.07, average removal of  $\text{NH}_4^+\text{-N}$ :average removal of  $\text{NO}_2^-\text{-N}$ :average generation of  $\text{NO}_3^-\text{-N}$  was about 1:1.28:0.18, which is close to the theoretical value of 1:1.32:0.26. This is seen from  $\text{NH}_4^+ + 1.32\text{NO}_2^- + 0.066\text{HCO}_3^- + 0.13\text{H}^+ \rightarrow 1.0\text{N}_2 + 0.26\text{NO}_3^- + 0.066\text{NH}_2\text{O}_{0.5}\text{N}_{0.15} + 2.03\text{H}_2\text{O}$ . Simultaneously,  $\text{NO}_3^-\text{-N}$  was below 2.5 mg/L, which indicates that good PN was established in the PN reactor. PN was maintained stably and nitrite accumulation was achieved.

The performance of the Anammox reactor is shown in Figure 3. The DO carried over from the PN reactor was removed, and the pH was adjusted. The flow rate was set to obtain an HRT of 4 h in the Anammox reactor. The influent ammonium and nitrite concentrations were in the range of 40–50 mg-N  $\text{L}^{-1}$ , respectively. The ratio was approximately 1:1.07. A consistent  $\text{NH}_4^+\text{-N}$  removal was observed and small quantities of  $\text{NO}_2^-\text{-N}$ , and  $\text{NO}_3^-\text{-N}$  accumulation were found in the Anammox reactor.

Average removal of  $\text{NH}_4^+\text{-N}$ :average removal of  $\text{NO}_2^-\text{-N}$ :average generation  $\text{NO}_3^-\text{-N}$  was about 1:1.28:0.18, which is close to the theoretical value of 1:1.32:0.26. The TN removal observed was around 79.9%. Good removal of TN was achieved, probably due to the proper ammonium and nitrite concentrations of about 1:1.07.

The sequential PN and Anammox process can be achieved in two separate reactors as in this study and the SHARON–Anammox process, or in a single oxygen controlled reactor. In this, nitrite production and the Anammox process simultaneously occur in a single oxygen controlled reactor as in the CANON process, which is a single oxygen controlled reactor. Two-reactor nitrification–Anammox processes are more effective for nitrogen removal than the one-reactor types. When the PN reactor and Anammox reactor are separated, the inhibitory effect of  $\text{O}_2$  on the Anammox reactor is relieved. Thus, each reactor's performance could be optimized more efficiently.

The variation in concentration and removal rate of COD over time are shown in Figure 4. The COD removal observed was around 75.0%, with the influent containing a nominal 150–200 mg/L COD. Good removal of TN and COD was achieved, this is probably due to the formation

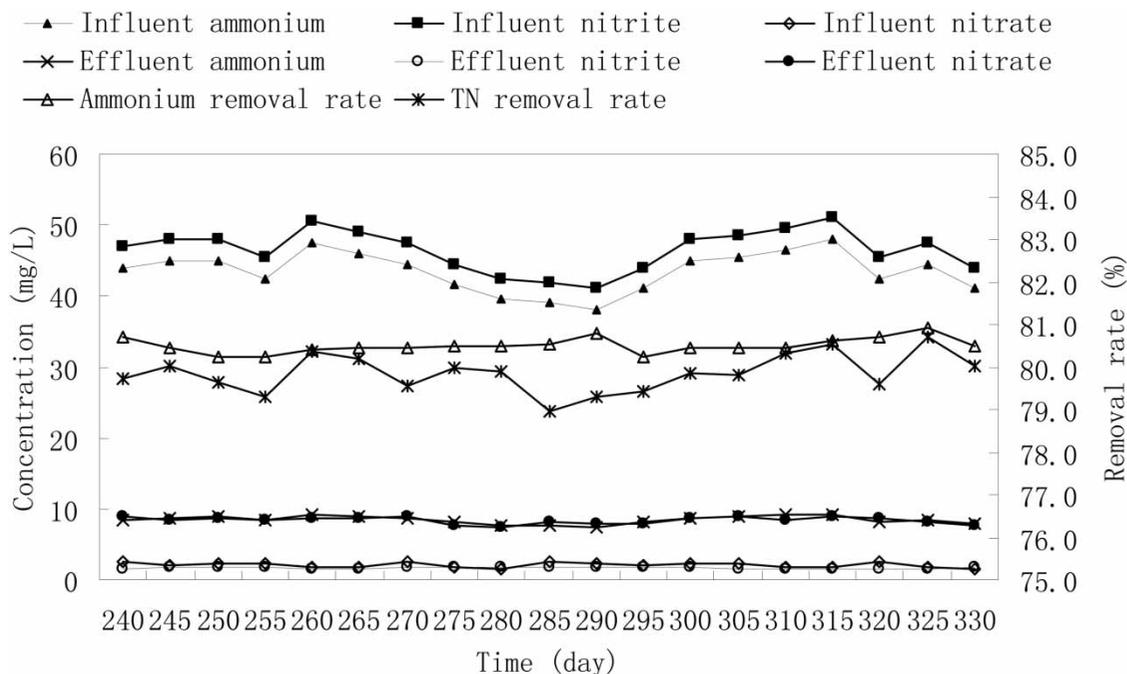
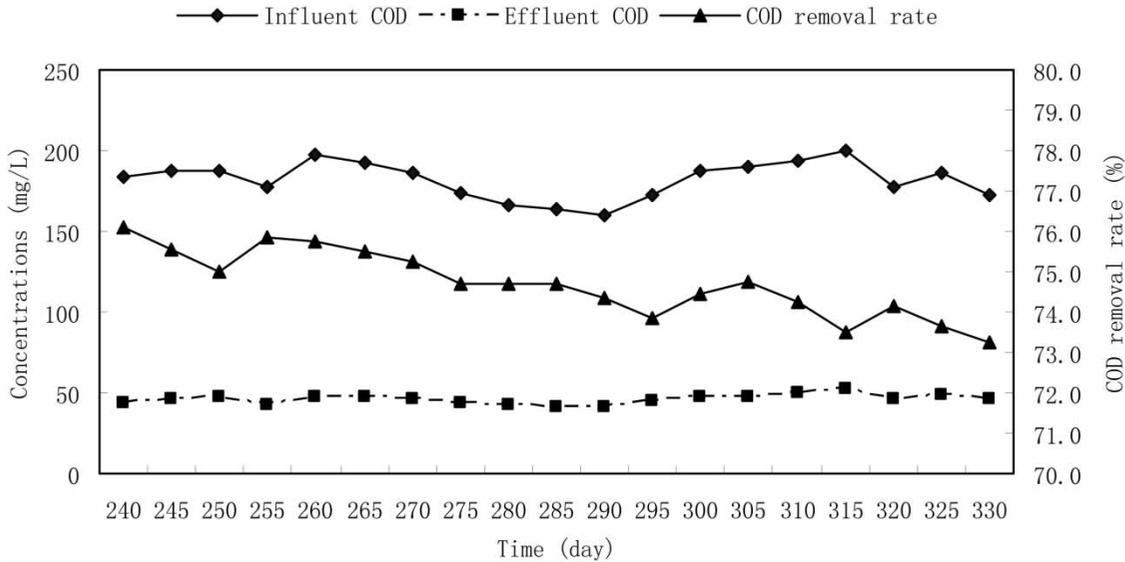


Figure 3 | Profiles of influent and effluent nitrogen concentrations, and their removal efficiencies in the Anammox process.



**Figure 4** | Profiles of influent and effluent COD and removal rate of over time.

of granular sludge which could sustain high microbial activity and retain the good performance of the PN-Anammox processes. Granular sludge was the key factor for the effective operation of PN-anammox processes. The reactor had good potential to treat aquaculture wastewater, and so had practical significance to study.

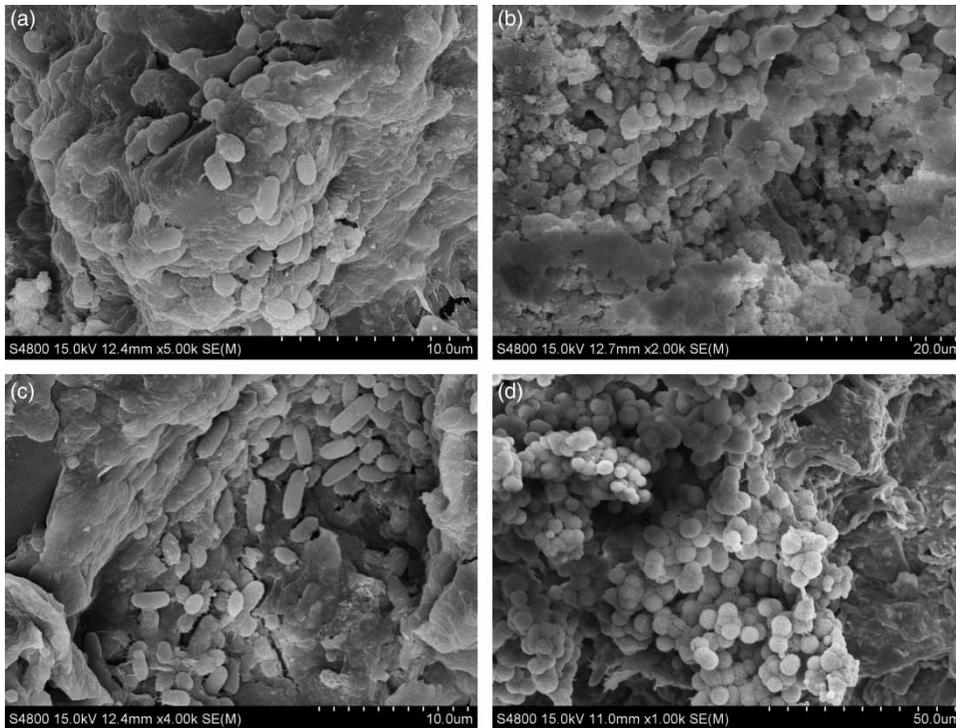
#### Microbiology characteristics of granular sludge in Anammox reactor

Samples on day 90 after start-up and on day 320 were observed by SEM observation. Microbial groups, including ammonia oxidizing bacteria (AOB) and Anammox bacteria, were investigated in this study. The SEM images of the granular sludge from the PN reactor and Anammox reactor are given in Figure 5(a)–5(d), respectively. Different bacterial morphologies were observed within the PN-Anammox reactor. Two groups of bacteria were distinguished by cell size and shape. The AOB cells were originally categorized into five genera on the basis of the shape of the cells and the arrangement of their intracytoplasmic membranes. However, as illustrated in Figure 5(c), spherical short rod-shaped *Nitrosomonas* predominantly existed in the PN reactor. The cell size was  $(0.7\text{--}1.5) \times (1.0\text{--}2.4) \mu\text{m}$  (under the ocular micrometer). As illustrated in Figure 5(d), the Anammox cells were round or oval shape, with cell size

being from 0.8 to 1.1  $\mu\text{m}$  (under the ocular microscope) in diameter. Anammox granular sludge showed a high degree of compactness, each cell being tightly integrated with others and they became microunits with little space between them.

Based on the analysis above, we could confirm the AOB and Anammox bacteria in the SEM photos easily. From the results, we concluded that the AOB and Anammox bacteria increased greatly compared to reactor start-up, the *Nitrosomonas* sp. consumed the oxygen and made a suitable and favorable growing environment for Anammox bacteria. On the other hand, Anammox bacteria could directly use the nitrite produced by the AOB bacteria and lessened the inhibition of nitrite on the AOB.

Molecular analysis allows an insight into the distribution and amount of the AOB and Anammox bacteria, potentially revealing a basis for autotrophic nitrogen removal. FISH analysis was performed with the samples taken from the PN reactor and the Anammox reactor on day 320, illustrated in Figure 6(a) and 6(b). We utilized the AOB bacteria (Nso190, Nsv443 and Nsm156) and anammox (Amx368) probes labeled with FITC (yellow) and Cy3 (red), respectively. Imaging using confocal laser scanning microscopy revealed a high density of cells growing in clusters and emitting red and yellow fluorescence, indicating the microbial composition of the granules (Figure 6(a)).

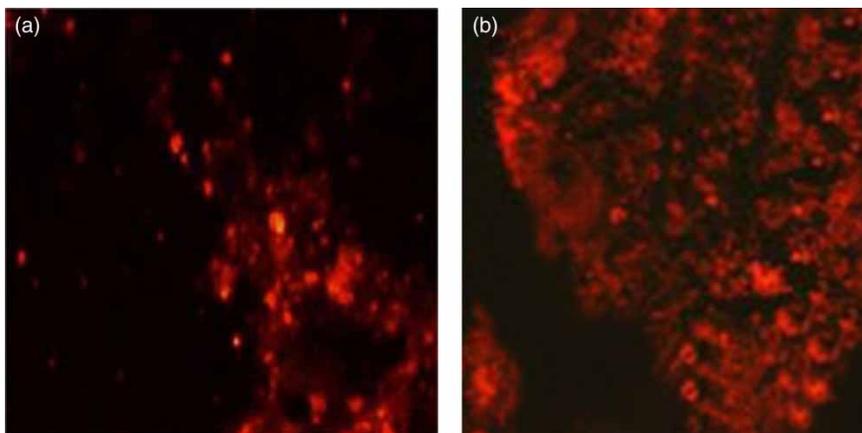


**Figure 5** | SEM showing the bacterium of granular sludge: (a) short rod-shaped *Nitrosomonas* predominantly existed in the PN reactor on day 90 after start-up; (b) round or oval shape Anammox cells existed in the Anammox reactor on day 90 after start-up; (c) short rod-shaped *Nitrosomonas* predominantly existed in the PN reactor on day 320; (d) round or oval shape Anammox cells existed in the Anammox reactor on day 320.

Hybridized AOB with the Cy3-labeled probe, including Nsm156, specific for *Nitrosomonas* spp., produced strong red signals (Figure 6(a)). The yellow signal of bacteria hybridized with two FITC labeled probes, including NSO190, specific for b-proteobacterial AOB, and Nsv443, specific for *Nitrospira* spp., was quite weak (Figure 6(a)). It

clearly indicated that AOB became the dominant nitrifying bacteria.

Based on the image of samples from day 320, hybridized Anammox bacteria with Cy3-labeled probe, including Amx368, produced strong red signals (Figure 6(b)). It clearly indicated that Anammox bacteria were the dominant



**Figure 6** | FISH showing the bacterium of granular sludge: (a) Image of the AOB cells hybridized with FITC (yellow) and Cy3 (red) labeled probes Nso190, Nsv443 and Nsm156 (red); (b) red color indicates anammox bacteria hybridized with Cy3-labeled Amx368 probe. The full color version of this figure is available in the online version of this paper: <http://dx.doi.org/10.2166/wrd.2016.156>.

bacteria in the reactor. The FISH image on day 320 elucidated more clearly the spatial location of the Anammox and AOB cells compared with SEM photos. In Figure 6(b), most Anammox cells agglomerated in groups, and in Figure 6(a), the AOB formed as relatively dispersed cells. This corresponds to the SEM images of each.

### Effect of FA and free nitrous acid on process of the PN-anammox reactor

As discussed above, by adjusting the pH, temperature, and the remaining concentration of ammonium, FA and FNA concentrations were calculated according to Equations (2) and (3) (Chen et al. 2010; Mousavi et al. 2014):

$$FNA\left(\frac{mg}{L}\right) = \frac{17}{14} \times \frac{[NH_4 \cdot N] \times 10^{pH}}{\exp[6344/(273 + T)] + 10^{pH}} \quad (2)$$

$$FNA\left(\frac{mg}{L}\right) = \frac{46}{14} \times \frac{[NO_2^- \cdot N]}{\exp[-2300/(273 + T)] + 10^{pH}} \quad (3)$$

According to the literature, FA and free nitrous acid (FNA) show inhibition on nitrification, especially from nitrite to nitrate oxidation. The accumulation of free FA and FNA has strong and complicated effects on the inhibition of Nitrobacter activity. FA begins to inhibit *Nitrosomonas* between 10 and 150 mg NH<sub>3</sub>/L, whereas Nitrobacter activity is already seriously reduced between 0.1 and 10 mg NH<sub>3</sub>/L (Fux et al. 2002). According to Equation (2), the FA concentration in this study was approximately 0.5–0.7 mg NH<sub>3</sub>/L. Hence, it can be concluded that the concentration of FA is a key factor for achieving nitrite accumulation and nitrite oxidizer inhibition. FNA begins to inhibit Nitrobacter activity when its concentration is 0.01 mg HNO<sub>2</sub>/L, whereas Nitrobacter almost stops growing when the concentration is 0.1 mg HNO<sub>2</sub>/L (Anthonisen et al. 1976). According to Equation (3), the FNA concentration in this study was approximately 0.08–0.1 mg HNO<sub>2</sub>/L. So the inhibition by FA and nitrous acid selectively suppressed the growth of nitrite oxidizers and washed them out of the PN reactor. The stable performance of the PN process was maintained with the highest nitrogen conversion rate.

At the same time, FA and FNA concentrations also inhibit the activity of Anammox bacteria. FA begins to inhibit Anammox bacteria when the concentration is 10 mg NH<sub>3</sub>/L, and Anammox bacteria are already seriously reduced when it reaches 100 mg NH<sub>3</sub>/L. Fortunately, the FA concentration in this study was approximately 0.5–3.2 mg NH<sub>3</sub>/L, while FA begins to inhibit Anammox bacteria when the concentration is 10 mg NH<sub>3</sub>/L. Therefore, the FA eliminated one inhibition on the activity of Anammox bacteria. In addition, the Anammox process is a process of alkali production. From Equation (1) it can be seen that H<sup>+</sup> is consumed, and the pH will increase. According to Equation (3), the FNA concentration in this study will further decrease with the increase in pH, which is good for the Anammox process. So the inhibition by FA and FNA which suppresses the growth of Anammox bacteria does not occur. The stable performance of the Anammox process was maintained with a high nitrogen conversion rate.

### CONCLUSIONS

1. Good removal of TN and COD was achieved, probably due to the formation of granular sludge, which could sustain high microbial activity and maintain the good performance of the PN-Anammox processes. The reactor had good potential to treat aquaculture wastewater.
2. In the PN reactor, the AOB were predominant in the interior of the granular sludge, which formed as relatively dispersed cells. Short rod-shaped *Nitrosomonas* predominantly existed in the PN reactor. Anammox granular sludge showed a high degree of compactness, each cell was tightly integrated with others. The Anammox cells were round or oval shaped, cell size was from 0.8 to 1.1 μm in diameter.
3. The FISH result indicated that AOB became the dominant nitrifying bacteria in the PN reactor. Nsm156, which is specific for *Nitrosomonas* spp., was predominant, while NSO190, which was specific for b-proteobacterial AOB and Nsv443, which was specific for *Nitrosospira* spp., were quite weak. In Figure 6(b), most anammox cells agglomerated in groups, and in Figure 6(a), the AOB formed as relatively dispersed cells. The FISH image on day 320 elucidated more clearly

the spatial location of Anammox and AOB cells compared with the SEM photos.

- The inhibition by FA and nitrous acid selectively suppressed the growth of nitrite oxidizers and washed them out of the PN reactor. The stable performance of the PN process was maintained at a high nitrogen conversion rate. Inhibition by FA and FNA of the growth of Anammox bacteria did not occur. The stable performance of the Anammox process was maintained with a high nitrogen conversion rate.

The results show that an understanding of the microbiological characteristics of immobilized granular sludge in PN-Anammox reactors is helpful for cultivating granular sludge, which ensures the effective running of the reactor.

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